Study Title

Activated Sludge, Respiration Inhibition Test with

PES Vorstufe 2342

Data Requirements / Test Guidelines

EU method C.11 (2008)

OECD TG 209 (1984)

Author:

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Study completion date:

2011-04-04

Sponsor:

Bayer MaterialScience AG

BMS-IO-ST-PSRA-GPRA

51368 Leverkusen

Germany

Testing facility:

CURRENTA GmbH & Co. OHG

Analytik

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Laboratory Project Identification

Study No. 2010/0087/12

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CONTENTS

Page

1. GLP DECLARATION

This study was conducted in compliance with the OECD principles of Good Laboratory Practice (1999) and with the Principles of Good Laboratory Practice according to Annex I, German Chemical Law (2008).

Date / Signature

Study Director 2011-04-04 A. Neuhahn
(Prof. Dr. ~~Caspers~~ / Neuhahn)

2. ARCHIVING

The original report, the study plan, and all raw data pertaining to this study are stored in the "GLP Archive, CURRENTA GmbH & Co. OHG, Analytik, CHEMPARK, Building Q 18, 51368 Leverkusen". A sample of the test item is stored in "GLP-Sample Store, CURRENTA GmbH & Co. OHG, Analytik, CHEMPARK, Building DA1, 41538 Dormagen".

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3. QUALITY ASSURANCE STATEMENT

This report was audited by the Quality Assurance Unit CURRENTA Analytik, Quality Management at CURRENTA GmbH & Co. OHG and this statement confirms that the final report reflects the raw data.

The dates of Quality Assurance inspections and audits are given below.

Audits	Dates of QAU inspections	Dates of reports
study plan review	2011-03-10	2011-03-10
process based inspection	2011-02-08 2010/0141/01	2011-02-08
final report review (draft)	2011-03-24	2011-03-24
final report review	2011-04-05	2011-04-05

Date / Signature

2011-04-05 A. Senic

(Senic/ ~~Dr. Dörzbach-Lange/ Dr. Neupert~~)

4. STUDY TIME TABLE

Study initiation date:	2011-03-10
Study completion date:	2011-04-04
Start of experimental phase:	2011-03-14
End of experimental phase:	2011-03-18

5. GLP CERTIFICATE

Ministerium für Umwelt und Naturschutz, Landwirtschaft und Verbraucherschutz
des Landes Nordrhein-Westfalen

Postanschrift: 40190 Düsseldorf

Aktenzeichen: VI-3- 31.11.65.05

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Bayer Industry Services GmbH & Co OHG
Prüfeinrichtung BIS-SUA-Analytics
D-51368 Leverkusen

(unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien (gemäß ChemVwV-GLP Nr. 5.3/OECD guidance)	Areas of Expertise (according ChemVwV-GLP Nr. 5.3/OECD guidance)
Kategorie 1	category 1
Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	physical-chemical testing
Kategorie 4	category 4
Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	environmental toxicity studies on aquatic and terrestrial organisms
Kategorie 5	category 5
Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung	studies on behaviour in water, soil and air; bioaccumulation
Kategorie 8	category 8
Analytische Prüfungen an biologischen Materialien	analytical and clinical chemistry testing

Datum der Inspektion (Tag.Monat.Jahr)	Date of Inspection (day.month.year)
14. bis 16. September und 26. bis 28. Oktober 2005	on 14 until 16 September and on 26 until 28 October 2005

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Düsseldorf, den 11. Januar 2006
Im Auftrag

(Prof. Dr. David)

Dienstsiegel/official-seal

Please note: Effective January 1st, 2008 the company name Bayer Industry Services GmbH & Co. OHG was changed to CURRENTA GmbH & Co. OHG

6. SUMMARY

A study was performed to assess the toxicity of PES Vorstufe 2342 to bacteria.

The study was conducted in accordance with Council Regulation (EC) No 440/2008, Method C.11 ”Biodegradation: Activated Sludge Respiration Inhibition Test” (2008). This test method is equal to OECD Guideline 209 (1984).

The activated sludge was exposed to PES Vorstufe 2342 at different concentrations. The respiration rate of each mixture was determined after aeration periods of 3 hours.

PES Vorstufe 2342 showed 0.0 % respiration inhibition of activated sludge at a test item concentration of 1000 mg/L.

The EC50 is higher than 1000 mg/L.

The effect value relates to a nominal concentration, since no analytical monitoring was performed.

7. EXPERIMENTAL PROCEDURE

The purpose of this test was to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified, and to indicate suitable non-inhibitory concentrations of test items to be used in biodegradability tests.
The method assessed the effect of a test item on microorganisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test item.

The study was conducted in accordance with Council Regulation (EC) No 440/2008, Method C.11 ”Biodegradation: Activated Sludge Respiration Inhibition Test” (2008). This test method is equal to OECD Guideline 209 (1984).

To measure the oxygen consumption, 250 mL of sludge with the test item (or control or reference compound) was incubated for 3 h in 300 mL closed Erlenmeyer flasks (with air inlet and outlet) and aerated through a glass tube at 50-100 L/h with clean oil-free air. For the measurement, the content of the Erlenmeyer flasks was completely transferred to 250 mL BOD bottles and oxygen content was measured with an oxygen meter (redox electrode) with writer.

Two controls without the test item were included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge was checked using 3,5-Dichlorophenol as a reference compound.

Since some substances may consume oxygen by chemical reactivity, a physico-chemical oxygen consumption control was carried out additionally. In order to be able to differentiate between physico-chemical oxygen consumption and biological oxygen consumption (respiration), at least the maximum concentration of the test item was tested without activated sludge.

The respiration rate for each concentration was determined graphically from the linear part of the curve of the oxygen content versus time. The inhibitory effect of the test item at a particular concentration is expressed as a percentage of the mean of the respiration rates of two controls. An EC_{50} value was calculated from the respiration rates at different test item concentrations using the statistics programme ToxRatPro Version 2.10 (released 2010-02-20).

8. MATERIALS AND METHODS

8.1 Sample description

Test item	:	PES Vorstufe 2342
Chemical name	:	Castor Oil, reaction product with Soybean Oil
CAS name	:	--
CAS number	:	--
EC/NLP number	:	919-697-6
Sample provided by	:	Bayer MaterialScience
Empirical formula	:	--
Molecular mass	:	-- g/mol
Structural formula	:	--
Batch number	:	LB06603520
Charge	:	--
Sample number	:	1199
Date of receipt	:	2010-04-27
Expiry date	:	2011-11-09
Purity	:	100 % (according to data of the sponsor)
Water solubility	:	0.0058 g/l
Vapour pressure	:	ca. 4 hPa at 20 °C

8.2 Reference compound

Reference compound	:	3,5-Dichlorophenol 97 % (Aldrich)
Purity	:	99.6 %
Batch number	:	04621CJ

8.3 Test organism and synthetic sewage feed

Type	:	mixed population of aquatic microorganisms (activated sludge)
Origin	:	aeration tank of a domestic sewage treatment plant (Municipal STP Cologne-Stammheim)
Date of collection	:	2011-03-14
Microbial inoculum	:	The sludge was settled and the supernatant was decanted. After centrifuging the sludge (15 min at 4500 rpm and 20 °C) the supernatant was decanted again. Approximately 1 g of the wet sludge was dried in order to calculate the amount of wet sludge to achieve a concentration of activated sludge of 3 g/L (dry weight) suspended solids. The calculated amount of sludge was dissolved in synthetic medium and then filled up to a defined end volume with deionised water.
Storage of sludge	:	aeration of the activated sludge at 20 ± 2 °C, daily fed with synthetic medium
pH of the suspension before application	:	7.7
Synthetic sewage feed	:	A synthetic waste water feed was prepared by dissolving the following amounts of substance per 1 litre of water. 16.0 g peptone 11.0 g meat extract 3.0 g urea 0.7 g NaCl 0.4 g $CaCl_2$ x $2H_2O$ 0.2 g $MgSO_4$ x $7H_2O$ 2.8 g K_2HPO_4
pH of the synthetic sewage feed	:	7.5 ± 0.5

8.4 Test system

Before use the wet weight/dry weight relationship of the activated sludge was determined by drying 10 mL of sludge suspension. Subsequently, a sludge suspension of 2 g (dry weight)/L was prepared. The pH of this suspension was measured and adjusted to 6-8.
8 mL of the synthetic medium and 100 mL of activated sludge were added to the dissolved test item. The mixture was filled up with deionised water to 250 mL and aerated at 20 ± 2 °C.
The exposure medium with the reference substance was prepared by adding 8 mL of the synthetic medium, 100 mL of activated sludge and a defined amount of the stock solution to achieve the test concentrations, and was filled up with deionised water to 250 mL and aerated at 20 ± 2 °C.
Control vessels (inoculated sample without test item) were prepared the same way.
Additional vessels to determine the physico-chemical oxygen consumption were prepared containing the test item, and the synthetic medium but no activated sludge.
Oxygen consumption was measured and recorded after an aeration time of 3 hours in all these vessels starting with control 1. Thereafter, temperature and pH were measured as well. Then the other test vessels were measured. Control 2 terminated the measurements.

8.5 Apparatus

Analytical balance

pH meter

Oxygen meter

Water bath for incubation

Various glass material: volumetric flasks, beakers, watch glasses, pipettes etc.

8.6 Pre-treatment of test item and reference compound

Direct weighings were prepared to give the different test item concentrations. The test item was added into Erlenmeyer flasks (incubation vessels) to about 130 mL deionised water and was stirred before testing (equilibration phase) overnight for 16 hours.
For the reference compound a stock solution at a concentration of 500 mg/L was prepared by dissolving 250 mg 3,5-Dichlorophenol in 5 mL of 1 N NaOH and diluting to 0.5 litre with deionised water. The pH was adjusted to pH 7 ± 0.5 with HCl.

8.7 Exposure conditions

Test item concentration/s	:	10, 100 and 1000 mg/L
Test item concentration in physico-chemical oxygen consumption control	:	1000 mg/L
Concentration of reference compound 3,5-Dichlorophenol	:	5, 10 and 20 mg/L

The test item and reference compound concentrations were not confirmed by analytical methods, they were based on nominal concentrations.

Test vessels	:	300 mL glass Erlenmeyer flasks
Method of application	:	direct weighing
Test concentration of the activated sludge	:	800 mg/L suspended solids
Test temperature	:	20 ± 2 °C
Stirring period of the test item before start of incubation time	:	16 hours
Incubation time	:	3 hours with permanent aeration

8.8 Applied SOPs and methods

00144 V.1 Activated sludge, Respiration Inhibition Test

Deviations : none

9. RESULTS

Table 1: Oxygen content, temperature and pH values during exposure phase (test item)

	Test item concen-tration [mg/L]	O_2 start [mg O_2/L]	O_2 end [mg O_2/L]	Time (start-end) [minutes]	Temp. [°C]	pH
Test item	10	5.3	2.5	5	20.3	7.9
	100	4.9	2.5	4	20.4	7.9
	1000	4.1	2.7	2	20.6	7.8
Control 1		5.9	2.6	7	19.6	7.9
Control 2		5.3	2.7	5	20.6	7.9
Physico-chemical oxygen consumption control	1000	8.1	8.1	8	20.5	7.5

Comments: none

Table 2: Oxygen content, temperature and pH values during exposure phase (reference compound)

	Reference compound concen-tration [mg/L]	O_2 start [mg O_2/L]	O_2 end [mg O_2/L]	Time (start-end) [minutes]	Temp. [°C]	pH
3,5-Dichloro-phenol	5	6.4	3.2	9	19.6	7.9
	10	7.0	5.3	9	19.7	7.8
	20	7.5	6.9	7	19.8	7.9

Comments: none

Table 3: Results of test item PES Vorstufe 2342

Test item concentration (nominal) [mg/L]	Respiratory rate test item [mg/L · h]	Phys.-chem. O_2 consumption [mg/L · h]	Respiratory rate - phys.-chem. O_2 consumption [mg/L · h]	Inhibition [%]
10	33.6	0.0	33.6	0.0
100	36.0	0.0	36.0	0.0
1000	42.0	0.0*	42.0	0.0

Control, mean	29.7
Control 1	28.3
Control 2	31.2

Comments: Concentrations are given as nominal concentrations and were not confirmed by analytical methods.
* The physico-chemical oxygen consumption has been determined at 1000 mg/L test item concentration. As no physico-chemical oxygen consumption was observed at that test item concentration this observation also holds true for the lower test item concentrations.

Table 4: Results of reference compound 3,5-Dichlorophenol

Reference compound concentration (nominal) [mg/L]	Respiratory rate reference compound [mg/L · h]	Inhibition [%]
5	21.3	28.3
10	11.3	61.9
20	5.1	82.7
Control, mean	29.7	
Control 1	28.3	
Control 2	31.2	

Comments: Concentrations are given as nominal concentrations and were not confirmed by analytical methods.

After an incubation period of 3 hours, analysis of the respiration rates gave the following values:

Results [mg/L]:

EC 50: > 1000

EC 10: > 1000

PES Vorstufe 2342 showed 0.0 % respiration inhibition of activated sludge at the highest test item concentration of 1000 mg/L.

The EC_{50}-determination of the reference compound is given in **figure 1**.

9.1 Comments

All validity criteria of the test method were met:

- respiratory rates of the 2 controls differ less than 15 % from each other
- the EC_{50} of the reference compound 3,5-Dichlorophenol is in the range 5 – 30 mg/L

Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95 %- and 99 %-confidence limits (according to Fieller`s theorem).

Parameter	EC10	EC20	EC50	EC80
Value [mg/l]	2.6	3.8	8.0	17.0
lower 95 %-cl	n.d.	n.d.	n.d.	n.d.
upper 95 %-cl	n.d.	n.d.	n.d.	n.d.
lower 99 %-cl	n.d.	n.d.	n.d.	n.d.
upper 99 %-cl	n.d.	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data

Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The effect parameters and confidence limits can be meaningless. Slope function after Litchfield and Wilcoxon: 2.433

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

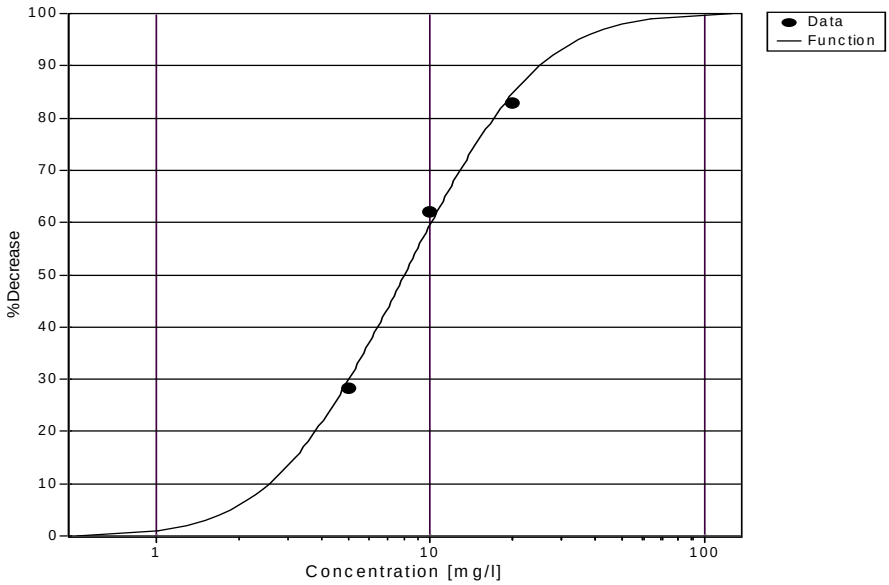

Figure 1: EC_{50}-determination of the reference compound 3,5-Dichlorophenol